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(54) Title: METHOD FOR CELL SURFACE DISPLAY OF TARGET PROTEINS USING FADL OF E. COLI

(57) Abstract: The present invention relates to an expression vector which can effectively express target proteins or peptides on the surface of cells using an outer membrane protein (FadL) of E. coli as a surface anchoring motif. Also, the present invention relates to microorganisms transformed with the expression vector, and a method for stably expressing large amounts of target proteins on the surface of cells by culturing the transformed microorganisms. Furthermore, the present invention relates to a production method of protein arrays, a production method of antibodies, and a bioconversion method, the methods being characterized by using target proteins which have been expressed on the cell surface by the inventive method. In addition, the present invention relates to a method for improving target proteins by the inventive surface expression method. The present invention allows target proteins with normal functions to be expressed on an outer cell membrane. Thus, the present invention will be useful in recombinant live vaccines, the screening of various peptides or antibodies, whole-cell adsorbents for heavy metal removal or waste water treatment, whole-cell bioconversion, and the like.

METHOD FOR CELL SURFACE DISPLAY OF TARGET
PROTEINS USING FadL OF *E. coli*

5

TECHNICAL FIELD

The present invention relates to a method for stably expressing large amounts of target proteins on the surface of cells using an outer membrane protein
10 (FadL) derived from gram-negative bacteria (*E. coli*). Also, the present invention relates to a production method of protein arrays, a production method of antibodies, and a bioconversion method, the methods being characterized by using target proteins which have been expressed on the cell surface by said method. In addition, the present invention relates to a method
15 for improving target proteins using the inventive surface expression method.

BACKGROUND ART

20 Cell surface display refers to a technology of expressing proteins or peptides in a form fused with a suitable surface anchoring motif on the surface of cells, such as gram negative and positive bacteria, molds, yeasts and animal cells (Lee, S.Y. *et al.*, *Trend. Biotechnol.*, 21:4552, 2003). In 1980s, the surface-expression system was first developed by expressing peptides or small proteins
25 fused with pIII of a filamentous phage with a relatively simple surface. Although the cell surface display using the phage was used in the screening of antibodies, epitopes, high-affinity ligands and the like, the size of proteins which can be expressed on the phage surface is relatively limited. Thus, as an alternative substitute therefor, a cell surface expression method for stably
30 expressing foreign proteins on the surface of microorganisms using a surface

protein of microorganisms, such as bacteria or yeasts, as a surface anchoring motif, has been developed.

In order to express a foreign protein on the surface of cells using the outer
5 membrane protein of a certain organism, a suitable surface protein and the
foreign protein should be linked with each other at a gene level to
biosynthesize a fusion protein, which should be stably passed through a cell
inner membrane and attached, and then maintained on the cell surface. For
this purpose, a protein having the following properties is preferably selected
10 for use as a matrix for surface expression. Namely, (1) it has a secretion
signal capable of passing through the cell inner membrane, at the N-terminal
end; (2) it must have a targeting signal which can be stably attached on the
surface of a cell outer membrane; (3) it can be expressed on the cell surface in
large amounts within range of having no adverse effect on the growth of cells,
15 such that the protein can show high activity; and (4) it is stably expressed
regardless of its size such that it should be able to be used in various reactions
(Georgiou *et al.*, *Trend. Biotechnol.*, 11:6, 1993). Such a matrix for surface
expression needs to be genetically engineered such that it is inserted into the
N-terminal end, C-terminal end or central portion of the outer membrane
20 protein on the surface of the host cells (Lee *et al.*, *Trend. Biotechnol.*, 21:45-
52, 2003).

In order to successfully express target peptide or protein on the surface of
bacteria, such as *E. coli* with a complex membrane structure, it is first required
25 to select a surface anchoring motif by which foreign proteins to be expressed
on the cell surface can be stably and efficiently transported to the cell surface.
Surface anchoring motifs which have been used in *E. coli* till now include
outer membrane proteins, lipoproteins, autotransporters, the subunits of surface
appendages, and S-layer proteins. Among them, the outer membrane proteins
30 have several advantages such as efficient secretory signal, unique membrane

spanning structures providing fusion sites, and strong anchoring structures. Owing to such advantages, the outer membrane proteins have been frequently used as a surface anchoring motif.

- 5 Outer membrane proteins such as OmpA, OmpS, LamB, OprF, PhoE and the like have widely been used for the expression of peptides, antibodies, domains, receptors, which have a relatively small molecular weight (Agterberg, M. *et al.*, *Gene*, 88:37, 1990; Lang, H. *et al.*, *Eur. J. Bacteriol.*, 267:163, 2000). Since the C-terminal and N-terminal ends of inserted foreign proteins must be
10 placed close to each other in three dimensions, these cell surface display system show low stability for the large protein. In fact, if LamB or PhoE is inserted with a foreign protein comprised of 50-60 or more amino acids, it will encounter limitations in its structure, which make it impossible to form a stable membrane protein. Also, *E. coli* porin (outer membrane protein) was used
15 only in epitopes or metal binding motifs other than proteins comprised of at most 150 amino acids (Stahl, S. *et al.*, *Trends Biotechnol.*, 15:185, 1997; Kjaergaard, K. *et al.*, *Appl. Environ. Microbiol.*, 66:10, 2000).

- Cell surface display using a bacterial secretion system is used in a very wide
20 range of applications. Also, it can be used in various applications depending on proteins or peptides which are expressed on the cell surface. The expression of a certain protein on the cell surface allows simple screening of peptides, antibodies or receptors (Francisco, J.A.R. *et al.*, *PNAS*, 91:10444, 1993), and the expression of antigen epitopes on the cell surface allows the
25 production of live vaccines which can show a powerful immune response. Also, a certain enzyme required in fine chemicals, agricultural chemicals or medicines, which have been expressed on the cell surface, may be used as a whole-cell biocatalyst, or proteins capable of degrading contaminants or adsorbing metal ions, which have been expressed on the cell surface, may be
30 used in bioremediation (Charbit, A. *et al.*, *Gene*, 70:181, 1988; Sousa, C. *et al.*,

J. Bacteriol., 180:2280, 1998; Richins, R. *et.al.*, *Nat. Biotechnol.*, 15: 984, 1997).

Various kinds of surface anchoring motifs have been reported till now.

- 5 However, targets which can be expressed on the cell surface by one surface anchoring motif are limited, so that the development of surface anchoring motifs which is different from each other, is needed to express various proteins on the cell surface.
- 10 FadL from *E. coli* which is a protein associated with the fatty acid metabolism of *E. coli* is involved in fatty acid transport. Regarding the membrane topology of the FadL protein, the N-terminal and C-terminal ends of the protein are found in the peripheral cytoplasm, 10 loops are out of the outer cell membrane, and 9 loops are out towards the peripheral cytoplasm (Cristalli *et*
- 15 *al.*, *Arch. Biochem. Biophys.*, 377: 324, 2000).

Since FadL is a protein of *E. coli* itself, it can easily express foreign proteins in a form fused with the foreign protein using *E. coli* as a host cell. Also, this protein has 10 external loops providing various points which can be fused,

20 thus, it is expected that the FadL protein can increase the possibility of cell surface expression of foreign proteins. Furthermore, the FadL protein has a stability that has been pointed out as a shortcoming of enzymes expressed on the cell surface in several surface expression systems. In other words, this protein has an advantage in that it allows the enzymes expressed on the cell

25 surface to maintain their activity for a long time period under various conditions (high temperature, high pH, organic solvent, etc.).

Accordingly, the present inventors have truncated a gene following the ninth loop expected to have the highest expression possibility of 10 loops which are

30 out of the outer cell membrane in the outer membrane protein, fused a foreign

protein (lipase) to the truncated loop position of *E. coli*, and expressed the lipase on the surface of cells. As a result, the present inventors confirmed that the lipase expressed on the cell surface is useful in bioconversion reactions, thus perfecting the present invention.

5

DISCLOSURE OF INVENTION

It is an object of the present invention to provide a surface expression vector of
10 a target protein containing not only a *fadL* gene encoding an *E. coli* outer membrane protein (FadL) but also a gene encoding a target protein.

Another object of the present invention is to provide microorganisms transformed with said surface expression vector, and a method for the cell
15 surface expression of a target protein, which is characterized by culturing the transformed microorganisms.

Still another object of the present invention is to provide a method for the production of protein arrays, a method for the production of antibodies in
20 vertebrate animals, and a bioconversion method, the methods being characterized by using cells which are produced by said expression method and have a target protein expressed on the surface thereof.

To achieve the above objects, in one aspect, the present invention provides a
25 vector for expressing a target protein on the surface of cells, the vector comprising a *fadL* gene encoding an *E. coli* outer membrane protein (FadL), an antibiotic-resistant gene, a promoter, a gene encoding a target protein, and a gene recombinant which is constructed to be expressed on the surface of the cell in a form fused with the FadL protein if the target protein-encoding gene is
30 expressed in a host cell.

In a preferred embodiment of the inventive surface expression vector for the target proteins, a linker is inserted into the middle portion of the *fadL* gene, and a target protein-encoding gene is inserted into the linker. Alternatively, the C-terminal end of the *fadL* is truncated, and the target protein-encoding gene is inserted into the position of the truncated C-terminal end. Alternatively, a base sequence following the ninth loop of the *fadL* gene is truncated, and the target protein-encoding gene is inserted into the position of the truncated base sequence.

10

In a preferred embodiment of the inventive surface expression vector, the target protein is a protein with a portion of amino acid sequence eliminated, or a protein mutated position-specifically, to facilitate the expression of the target protein on the surface. The promoter is preferably a Tac promoter or a *gntT104* promoter, and it is obvious to a person skilled in the art that the target protein can be expressed by a suitable promoter whose expression can be induced in a host cell, by a promoter of the target protein gene, or by other suitable promoters which can be expressed in host bacteria.

15

20 In another aspect, the present invention provides microorganisms transformed with the above-described surface expression vector. In the present invention, microorganisms used for the transformation are modified, such that an extracellular or intracellular protease that degrades the target protein, cannot be produced, facilitate the cell surface expression of the target protein. Also, the
25 microorganisms are preferably bacteria.

In still another aspect, the present invention provides a method for the cell surface expression of a target protein, the method comprising the steps of culturing said transformed microorganisms to express a target protein on the
30 cell surface of the microorganisms, and collecting the cells having the target

protein expressed on the surface thereof.

In yet another aspect, the present invention provides a bioconversion method characterized by using the cells which are produced by said method and have a
5 target protein with enzymatic activity expressed on the surface thereof. As a catalyst substance for bioconversion reaction in this method, any biosubstance which can catalyze chemical reactions can be used after fusion expression with a FadL protein. Examples of the catalyst substance include enzymes and catalytic antibodies.

10

In a further aspect, the present invention provides a method for producing protein arrays, characterized by immobilizing the cells, which are produced by said method and have a target protein expressed on the surface thereof, on the surface of a substrate.

15

A protein array, arrays various proteins such as a DNA array or a DNA chip, particularly antibodies, on a solid surface, so that it provides a means capable of analyzing the expression and expression level of the desired target protein in certain cells. To prepare a protein array, proteins to be arrayed, should be
20 secured and immobilized on a solid surface. In an analysis process using the protein array, in order to bind immobilized proteins and to wash unbound proteins, various treatments, such as heating and changes in salt concentration and pH, are conducted, thus, the immobilization of a stabilized protein capable of resisting this severe environment is required. However, in cloning several
25 thousand to tens of thousands of genes into an expression vector, and expressing and separating them and then immobilizing them on a solid surface, many operations should be conducted in a repeated manner. Thus, such operations need to be conducted in a more simple and rapid manner.

30 The method for producing the protein array according to the present invention

provides a means allowing such operations to be conducted most easily. For the production of the protein array according to the present invention, the method which is conventionally used in the art can be applied (WO 00/61806; WO 00/54046; US 5,807,754; EP 0818467; WO 97/42507; US 5,114,674; and
5 WO 96/35953). The protein array produced by the inventive method can be used in a diagnostic kit, gene expression analysis, the analysis of protein-protein, protein-ligand or antigen-antibody interactions, metabolic process analysis, the screening of a new enzyme or improved enzyme, combinatorial biochemical synthesis, and biosensors.

10

Examples of solid substrates which can be used in the inventive production method of the protein arrays include glass (e.g., glass having functional groups exposed to the outside), Si, Ge, GaAs, GaP, SiO, SiN₄, modified silicon nitrocellulose, polyvinylidene fluoride, polystyrene, polytetrafluoroethylene,
15 polycarbonate, nylon, fiber, and a combination thereof. A linker molecule for protein immobilization may also be attached to the substrate, in which non-spotted portions of the substrate is preferably blocked. Meanwhile, the amount of the cells expressed according to the present invention, which are applied to each of the spot (or address), is determined depending on the form
20 of the arrays. An interaction between a sample and the inventive surface-expressed protein immobilized on the solid substrate may be either detected using the inherent characteristics of the protein (e.g., immune reactivity), or detected by a change in the signal of a suitable marker substance (e.g., fluorescent substance, luminescent substance, radioactive substance or epitope)
25 linked to the protein. The analysis of results obtained by the inventive protein arrays can be performed by an automated system which is known as "scanner" or "reader" in the art.

In still another aspect, the present invention provides a method for preparing
30 antibodies in vertebrate animals, the method comprising the steps of:

administering the cells which are produced by said method and have antigens expressed on the surface thereof, to the vertebrate animals except for human beings, thereby inducing an immune response in the vertebrate animals; and collecting antibodies produced by the immune response.

5

The surface expression technology as described above expresses an antigen or a part thereof on the cell surface to produce an antibody so that it provides a transfer means of recombinant live vaccine using the same. In the vaccines developed till now, attenuated pathogenic bacteria or viruses were mainly
10 used, and in the case of the bacteria, an antigen was secreted and expressed inside a cell, on a cell membrane, or outside a cell, and transferred to a host cell. Since the surface-expressed live vaccine shows a very strong immune response and can express an antigen continuously while growing in a host cell, it is noticed as a new vaccine transfer means. Particularly, when a pathogen-
15 derived antigenic epitope is expressed on the surface of non-pathogenic *E. coli* or *Salmonella* sp. and orally administered alive, it is known to show far more lasting and strong immunity, so that it can be used as a method for inducing antigen-antibody production (Georgiou *et al.*, *Nature Biotechnol.*, 15:29, 1997; and Lee *et al.*, *Nature Biotechnol.*, 18:645, 2000).

20

Martineau *et al.*, reported a very simple method of producing an antipeptide antibody using the expression technology on the *E. coli* surface (Martineau *et al.*, *Bio/Technol.*, 9:170, 1991). In this surface expression technology, the desired peptide is expressed on the surface protrusions of MalE and LamB
25 which is a cell outer membrane, and then, the whole cell or ground cell is administered to an animal to induce the production of an antipeptide antibody. According to this method, the antibody can be produced without chemically synthesizing peptide or attaching it to a transfer protein.

30 The cells having antigens expressed on the surface thereof, which are used in

the inventive production method of antibodies, are preferably safe cells for a subject to be administered, and preferably contain an adjuvant, such as incomplete or complete Freud's adjuvant. Meanwhile, the cells are preferably administered by injection, and more preferably an intravenous, celiac, 5 subcutaneous or intramuscular administration. In order to obtain a sufficient amount of the antibodies, the booster administration of the cells is preferably performed at a suitable time after the first administration of the cells.

In yet another aspect, the present invention provides a method for producing a 10 chiral compound, which comprises optically resolving racemic compounds into chiral compounds by enantioselective target protein, the method being characterized by using the lipase expressed on the surface of the cells produced by said method.

15 In further another aspect, the present invention provides a method for improving a target protein, the method comprising the steps of: (a) constructing a mutant library of a gene encoding a target protein; (b) constructing a gene recombinant which contains the gene mutant library of the target protein and a *fadL* gene to express the mutant of the target protein in a 20 form fused with a FadL protein; (c) transforming a host cell with either the gene recombinant or a vector containing the gene recombinant, the host cell being selected from the group consisting of gram negative bacteria, gram positive bacteria, actinomyces, yeasts and molds; (d) culturing the transformed host cells to express the gene mutant library on the cell surface; and (e) 25 screening the cells on which a target protein with improved characteristics have been expressed.

In the method for the improvement of a target protein according to the present invention, the gene library can be obtained by modifying the gene of a wild- 30 type target protein using DNA shuffling (Stemmer, *Nature*, 370:389, 1994),

StEP (Zhao, H. *et al.*, *Nat. Biotechnol.*, 16: 258, 1998), RPR (Shao, Z. *et al.*, *Nucleic Acids Res.*, 26:681, 1998), molecular breeding (Ness, J.E. *et al.*, *Nat. Biotechnol.*, 17:893, 1999), ITCHY (Lutz, S. *et al.*, *Cur. Opi. Biotechnol.*, 11:319, 2000), error-prone PCR (Cadwell, R.C. *et al.*, *PCR Method Appl.*, 2:28, 5 1992), and point mutation (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y., 1989), but is not limited thereto.

In the target protein improvement method of the present invention, the screening step is characterized by using the activity of a target protein, a 10 protein recognizing substance labeled to a target protein, a labeled ligand binding to a target protein, or an antibody binding specifically to a target protein. Also, the screening step can be conducted by using a flow cytometer (Georgiou, G., *Adv. Protein Chem.*, 55:293, 2000), and in the case of using the protein activity, the screening can be achieved by either measuring the growth 15 of a host in which a protein is expressed, or measuring the color development reaction catalyzed by the protein.

In the case of using the inventive protein improvement method having the above characteristics, an enzyme catalyzing chemical reaction that does not 20 occur biologically (e.g., Diels-Alder condensation), an enzyme with unnatural stereoselectivity or regioselectivity, an enzyme capable of catalyzing reaction in an organic solvent or a higher solution than organic solvent-aqueous solution, and an enzyme catalyzing reaction in extreme conditions such as high temperature and high pressure, etc., which cannot be easily obtained by the 25 prior method, can be rapidly obtained from wild-type enzymes.

The inventive method can be applied to all proteins. For example, the inventive method can be used for the surface expression and improvement of a target protein selected from the group consisting of hormones, hormone 30 analogues, enzymes, enzyme inhibitors, signaling proteins or parts thereof,

antibodies or parts thereof, single chain antibodies, binding proteins, binding domains, peptides, antigens, adhesion proteins, structural proteins, regulatory proteins, toxin proteins, cytokines, transcriptional regulators, blood coagulation factors, and plant defense-inducing proteins.

5

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows recombinant plasmids pTrcFadLPL and p104FadLPL according
10 to the present invention.

FIG. 2 shows a confocal microscopy photograph for lipase expressed on the surface of cells.

15 **FIG. 3** shows a plate photograph for proving the activity of lipase expressed on the surface of cells.

DETAILED DESCRIPTION OF THE INVENTION

20

The present invention will hereinafter be described in further detail by examples. It will however be obvious to a person skilled in the art that these examples are given for illustrative purpose only, and the scope of the present invention is not limited to or by these examples.

25

Although a bioconversion reaction using lipase is particularly illustrated in the following examples, it will be obvious to a person skilled in the art from the disclosures herein that the inventive method may utilize any enzyme expressed on a cell surface, such as beta-galactosidase, protease, cellulase,
30 glycosyltransferase, oxidoreductases, and aldolase. Also, the inventive

method may be applied to the case where the bioconversion reaction is monostep or multistep and be applied even to the case where the bioconversion reaction occurs in an aqueous or non-aqueous phase. Cells having enzymes expressed on the surface thereof may be used in an immobilized or non-immobilized state, and the bioconversion may be used in combination with other microorganisms or enzymes.

Example 1: Construction of recombinant vector (pTrcFadL)

10 In order to express an outer membrane protein on Trc promoter, a pTrcFadL which is a recombinant plasmid containing a trc promoter was prepared.

In order to obtain *E. coli* outer membrane protein (FadL) gene from which the C-terminal end has been removed, 30-cycle PCR on the chromosomal DNA of *E. coli* W3110 (ATCC 39936) as a template was performed with primers of SEQ ID NO: 1 and SEQ ID NO: 2. In the PCR reaction, each PCR cycle consisted of first denaturation for 5 minutes at 95°C, second denaturation for 30 seconds at 95°C, annealing for 1 minute at 50°C, and extension for 1 minute and 30 seconds at 72°C.

20

SEQ ID NO: 1: 5-ggaattcatggtcatgagccagaaaacc

SEQ ID NO: 2: 5-gctctagaacgattctgtgcaggaac

From a fragment obtained by the PCR reaction, a DNA fragment with the size of about 1100bp was separated by agarose gel electrophoresis, and cut with restriction enzymes *EcoRI* and *XbaI*. The cut fragment was linked with a DNA fragment which has been obtained by cutting trc promoter-containing plasmid pTrc99A (Pharmacia Biotech., Uppsala, Swede) with restriction enzymes *EcoRI* and *XbaI*, thus obtaining recombinant plasmid. The resulting recombinant plasmid was transformed into *E. coli* XL10-Gold by

electroporation, and then the transformed strains were selected on an LB plate medium containing 50 µg/L of ampicillin, thereby obtaining a pTrcFadL recombinant plasmid (see FIG. 1).

5 **Example 2: Preparation of recombinant vector (p104FadL)**

In order to express an outer membrane protein on a constitutive promoter, recombinant plasmid p104FadL containing a gntT104 promoter was prepared. Namely, a DNA fragment obtained by cutting gntT104 promoter-containing
10 plasmid p10499a (Park, S.J. *et al.*, *FEMS Microbiol. Lett.*, 214:217, 2002) with restriction enzymes *EcoRI* and *XbaI* was linked to the *E. coli* outer membrane protein (FadL) gene from which the C-terminal end has been removed, obtained in Example 1, thus constructing a recombinant plasmid. The obtained recombinant plasmid was transformed into *E. coli* XL10-Gold
15 (Stratagene Co.) by electroporation. The transformed strains were selected on an LB plate medium containing 50 µg/L of ampicillin, thus obtaining a p104FadL recombinant plasmid (see FIG. 1).

20 **Example 3: Preparation of recombinant expression vector (pTrcFadLPL and p104FadLPL)**

In order to prepare a recombinant plasmid which expresses lipase on the surface of cells, the lipase gene of *Pseudomonas fluorescens* was first constructed in the following manner. Namely, 30-cycle PCR on the
25 chromosomal DNA of *Pseudomonas fluorescens* as a template was performed with primers of SEQ ID NO: 3 and SEQ ID NO: 4. In the PCR reaction, each PCR cycle consisted of first denaturation for 5 minutes at 95°C, second denaturation for 45 seconds at 95°C, annealing for 45 seconds at 60°C, and extension for 1 minute and 30 seconds at 72°C.

30 SEQ ID NO: 3: 5-gctctagaatgggtgtatttgactacaagaac

SEQ ID NO: 4: 5-cccaagctttcaactgatcagcacacc

From the DNA fragment obtained by the PCR reaction, a lipase gene, a DNA fragment with the size of about 1.4kbp, was obtained by agarose gel electrophoresis. The lipase gene was cut with *Xba*I and *Hind*III, and inserted into each of pTrcFadL and p104FadL, thus preparing recombinant expression vectors pTrcFadLPL and p104FadLPL, respectively. The obtained vectors were transformed into *E. coli* XL10-Gold. The transformed strains were screened in an LB plate medium containing 50 µg/L of ampicillin, and the screened strain was cultured in an LB liquid medium, and then stored in a freezer at -80°C (see FIG. 1).

Example 4: Cell surface expression of lipase

The transformed strain XL10-Gold (pTrcFadLPL) prepared in Example 3 was inoculated into 10 ml of an LB liquid medium containing 50 µg/L of ampicillin and cultured at 37°C. When the absorbance at a 600nm wavelength by a spectrophotometer reached 0.5, 0.1mM IPTG was added to the medium so as to induce gene expression. 5 hours after the gene expression induction, the culture broth was centrifuged at 4°C and 6000 rpm for 5 minutes to collect cells, and the collected cells were washed with phosphate buffered saline solution (PBS, pH 7.2), and then re-suspended in a PBS solution containing 3 wt% bovine serum albumin (BSA). Rat anti-lipase serum, a primary antibody, was added to the 3 wt% BSA-containing PBS solution at a ratio of 1:1000, and then cultured at 4°C for 4 hours. The culture medium was washed five times with PBS solution, after which Texas Red-conjugated secondary antibody (goat anti-rat IgG) was added to the resulting culture medium at a ratio of 1:3000 and then cultured at 4°C for 12 hours. In order to remove unreacted secondary antibody, the culture medium was washed five times with PBS solution, and observed under a confocal microscope (Carl

Zeiss, Jena, Germany). The observation results showed that lipase was expressed on the cell surface (see FIG. 2).

Example 5: Activity of lipase expressed on cell surface

5

The transformed strain XL10-Gold (p104FadLPL) prepared in Example 3 was cultured in an LB plate medium containing 50 µg/L of ampicillin and 1% (v/v) of tributyrin. After 72 hours, halo was produced in the medium due to the activity of lipase (see FIG. 3).

10

Example 6: Optical resolution of racemic mandelic acid methyl ester using lipase expressed on cell surface

The transformed strain XL10-Gold (pTrcFadLPL) prepared in Example 3 was
15 inoculated again in 250mL Erlenmeyer flask including 100mL of LB liquid medium containing 50µg/L of ampicillin, and then cultured at 37°C. Gene expression was induced by adding 0.1mM of IPTG, when absorbance was 0.5 at 600nm wavelength by spectrophotometer. 5 hours after inducing the expression, culture broth was centrifuged at 4°C, 6000rpm for 5 minutes to
20 collect cells, and then, the collected cells were washed with 50mM Tris-HCl solution (pH 8.0), followed by resuspending in 3mL of buffer. Said re-suspension was put into a 10mL flask, to which 30 mg of racemic mandelic acid methyl ester was added and allowed to react for 48 hours with stirring. Then, the suspension was centrifuged at 4°C and 6000 rpm for 5 minutes to
25 remove cells. The supernatant was collected and adjusted to a pH of 3.0. After extraction of the supernatant with 10mL of ethyl acetate and hexane, the extract was analyzed by liquid chromatography. The analysis results showed that (S)-mandelic acid and (R)-mandelic acid methyl ester were obtained.

HPLC analysis conditions:

30

column: Chiralcel OD-H;

flow rate: 0.5 mL/min;

solvent: hexane : iso-propanol : tri-fluoroacetic acid = 90:10:0.1
(volume ratio);

detect: UV 254nm.

5

Example 7: Optical resolution of racemic β -lactam, racemic cis-3-acetoxy-4-phenylazetidin-2-one using lipase expressed on cell surface

Cells were collected in the same manner as in Example 6, and suspended in
10 3mL of 0.1M sodium phosphate buffer (pH 6.8). 20 mg of racemic β -lactam and racemic cis-3-acetoxy-4-phenylazetidin-2-one were added to the suspension and allowed to react for 24 hours with stirring. At this time, the reaction sample was applied to thin film chromatography (TLC) [chloroform : ethyl acetate : hexane = 3:2:3(volume ratio)] so as to check the progression of
15 the reaction.

After completion of the reaction, the resulting material was centrifuged at 6000 rpm for 7 minutes to remove cells. The supernatant was collected and extracted five times with 10mL of ethyl acetate. The solvent was removed
20 with a vacuum rotary distillation/concentration device, and the remaining material was applied to silica gel column chromatography. As a result, chiral β -lactam, 8 mg of (-)-cis-3-hydroxy-4-phenylazetidin-2-one (40% yield) and 6 mg of (-)-cis-3-acetoxy-4-phenylazetidin-2-one (30% yield) were obtained.

(-)-cis-3-hydroxy-4-phenylazetidin-2-one

25

[α]_D²⁰ -13.0 (c 0.5, CH₃OH);

¹H NMR (CDCl₃ & DMSO-d₆) δ 3.60(s, 1 H, OH),

4.95(d, J = 4.7Hz, 1H, C3 H), 5.88(d, J = 4.7Hz, 1H, C4H),

6.22(s, 1H, NH), 7.27-7.40(m, 5H, ArH);

¹³C NMR(CDCl₃ & DMSO-d₆) δ 58.6, 79.1, 127.6, 128.1, 137.1,

30

170.5;

CIMS, m/z 163(M⁺), 91(base).

(-)-cis-3-acetoxy-4-phenylazetidin-2-one

[α]_D-30.0 (c 1, CHCl₃); IR (KBr) 3200, 1750, 1720 cm⁻¹

- 5 1H NMR(CDCl₃) δ 1.68(s, 3H, CH₃CO),
5.05(d, J = 4.5Hz, 1H, C₃H),
5.88(dd, J = 2.6 & 4.5 Hz, 1H, C₄H),
6.25(s, 1H, NH), 7.29-7.39(m, 5H, Ar);
13C NMR(CDCl₃) δ 19.7, 57.9, 78.3, 127.5, 127.7, 128.2, 128.5,
10 134.7, 165.6((β -lactam CO), 169.0(acetoxy CO);
CIMS, m/z 205(M⁺), 106(base).

Example 8: pH stability of lipase expressed on cell surface

- 15 Cells were collected in the same manner as in Example 6, and suspended in 3mL of Tris-HCl buffer (pH 10.0). 100 μ l of the suspension was taken every hour and measured for activity. The results showed that more than 90% of the initial activity was maintained even after 48 hours. The activity measurement was conducted in the following manner. The taken sample was
20 centrifuged at 4°C and 6000 rpm for 5 minutes to collect cells, and the collected cells were suspended in a solution prepared by mixing a solution of 10mM p-nitrophenyl decanoate in acetonitrile, ethanol and Tris-HCl buffer (pH 8.0), at a volume ratio of 1:4:95, and the suspension was allowed to react at 37°C for 10 minutes. After the reaction was terminated by the addition of 2
25 μ l of 0.5M EDTA solution, the activity was calculated by measuring the absorbance at 405nm.

Example 9: Temperature stability of lipase expressed on cell surface

- 30 Cells were collected in the same manner as in Example 6, and suspended in

3mL of Tris-HCl (pH 8.0). The suspension was left as it was at 50°C, and measured for the activity. The results showed that more than 90% of the activity was maintained even after 120 hours.

5 **Example 10: Organic solvent stability of lipase expressed on cell surface**

Cells were collected in the same manner as in Example 6, and suspended in 3mL of hexane. The suspension was left as it was at 37°C and measured for the activity. The results showed that the activity was maintained for 48 hours.

10

As confirmed in Examples 6 to 10, lipase which has been normally expressed on the cell surface by the inventive method may be used in the preparation of chiral compounds. Also, it is easy to recover the lipase and its stability is excellent, so that it can make the preparation process of chiral compounds
15 simple and can increase the productivity in the preparation process of chiral compounds.

While the present invention has been described with reference to the particular illustrative embodiments, it is not to be restricted by the embodiments but only
20 by the appended claims. It is to be appreciated that those skilled in the art can change or modify the embodiments without departing from the scope and spirit of the present invention.

25

INDUSTRIAL APPLICABILITY

As described above, the present invention provides the expression vector which can effectively express target proteins or peptides on the surface of cells using an outer membrane protein (FadL) of *E. coli* as a surface anchoring
30 motif, microorganisms transformed with the expression vector, and a method

for stably expressing large amounts of target proteins on the surface of cells by culturing the transformed microorganisms.

The present invention allows target proteins with normal functions to be
5 expressed on the outer membrane of cells. Thus, the present invention will be useful in recombinant live vaccines, the screening of various peptides or antibodies, whole-cell adsorbents for heavy metal removal or waste water treatment, whole-cell bioconversion, and the like depending on target proteins inserted.

10

THE CLAIMS

What is Claimed is:

- 5 1. A surface expression vector of a target protein, comprising a *fadL* gene encoding an *E. coli* outer membrane protein (FadL), an antibiotic-resistant gene, a promoter, a gene encoding a target protein, and a gene recombinant which is constructed, to be expressed on the surface of the cell in a form fused with the FadL protein if the target protein-encoding gene is expressed in a host
10 cell.
2. The surface expression vector of a target protein according to claim 1, wherein a linker is inserted into the middle portion of the *fadL* gene, and the target protein-encoding gene is inserted into the linker.
- 15 3. The surface expression vector of a target protein according to claim 1, wherein the C-terminal end of the *fadL* is truncated, and the target protein-encoding gene is inserted into the position of the truncated C-terminal end.
- 20 4. The surface expression vector of a target protein according to claim 1, wherein a base sequence following the ninth loop of the *fadL* gene is truncated, and the target protein-encoding gene is inserted into the position of the truncated base sequence.
- 25 5. The surface expression vector of a target protein according to claim 1, wherein the target protein is a protein with a portion of amino acid sequence eliminated, or a protein mutated position-specifically, to facilitate the expression of the target protein on the surface.
- 30 6. The surface expression vector of a target protein according to claim 1,

wherein the promoter is a trc promoter or a gntT104 promoter.

7. A microorganism transformed with the surface expression vector of any one claim among claims 1 to 6.

5

8. The transformed microorganism according to claim 7, wherein the microorganism used for the transformation is modified such that an extracellular or intracellular protease that degrades the target protein, cannot be produced, to facilitate the cell surface expression of the target protein.

10

9. The transformed microorganism according to claim 7, wherein the microorganism is bacterium.

10. The transformed microorganism according to claim 9, wherein the
15 bacterium is *E. coli*.

11. A method for the cell surface expression of a target protein, the method comprising the steps of: culturing the transformed microorganism of any one claim among claims 7 to 10, to express a target protein on the cell surface of
20 the microorganisms; and collecting the cells having the target protein expressed on the surface thereof.

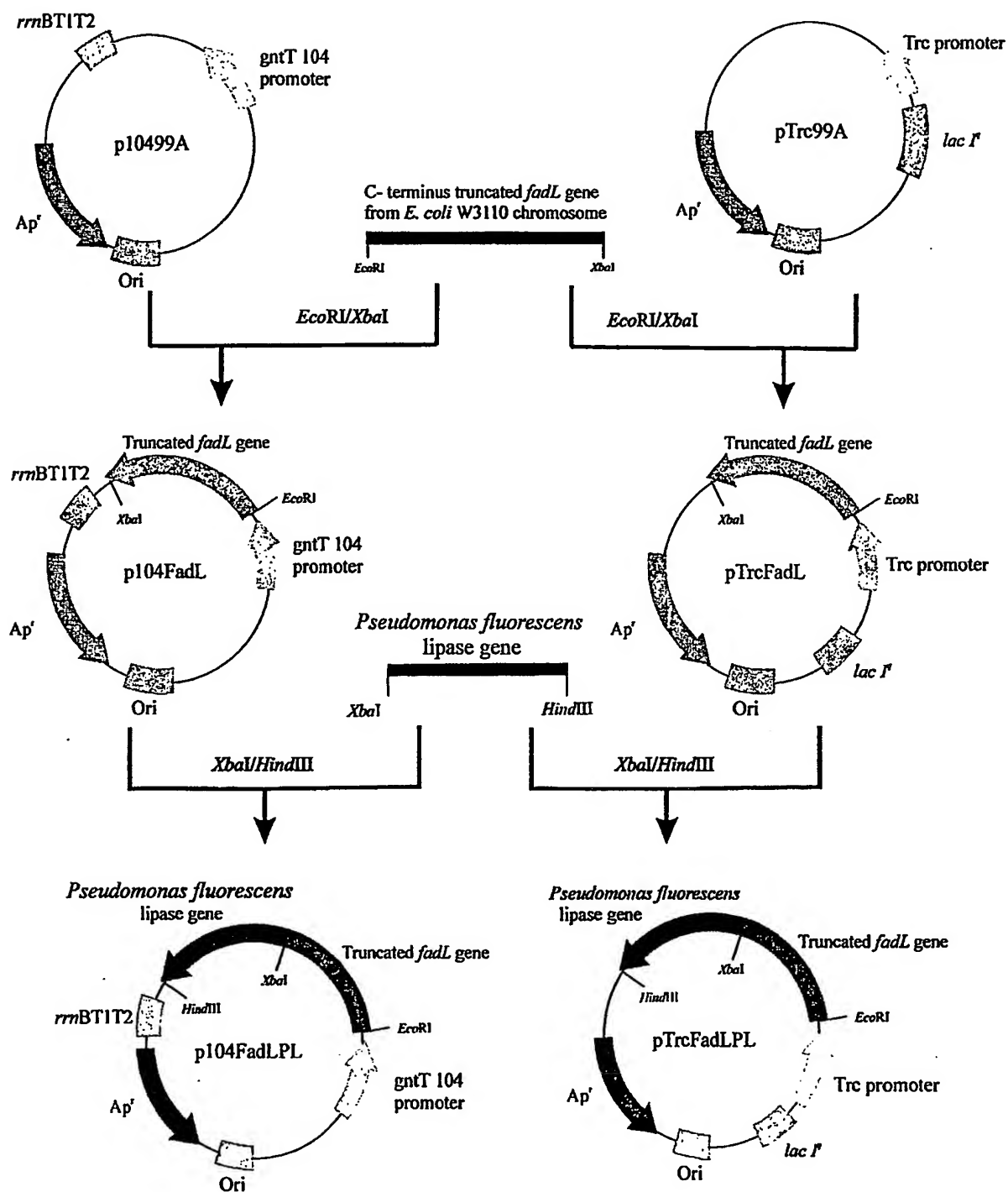
12. The method for the cell surface expression of a target protein according to claim 11, wherein the target protein is a protein selected from the group
25 consisting of hormones, hormone analogues, enzymes, enzyme inhibitors, signaling proteins or parts thereof, antibodies or parts thereof, single chain antibodies, binding proteins, binding domains, peptides, antigens, adhesion proteins, structural proteins, regulatory proteins, toxin proteins, cytokines, transcriptional regulators, blood coagulation factors, and plant defense-
30 inducing proteins.

13. The method for the cell surface expression of a target protein according to claim 12, wherein the enzyme is lipase.
- 5 14. A bioconversion method, characterized by using the cells which are produced by the method of claim 12, and have a target protein with enzymatic activity expressed on the surface thereof.
- 10 15. A method for producing protein arrays, characterized by immobilizing the cells which are produced by the method of claim 12, and have a target protein expressed on the surface thereof, on the surface of a substrate.
- 15 16. A method for preparing antibodies in vertebrate animals, the method comprising the steps of: administering the cells which are produced by the method of claim 12, and have antigens expressed on the surface thereof, to the vertebrate animals except for human beings, thereby inducing an immune response in the vertebrate animals; and collecting antibodies produced by the immune response.
- 20 17. A method for producing a chiral compound, which comprises optically resolving a racemic ester compound into chiral ester, chiral organic acid or chiral alcohol by lipase, the method being characterized by using the lipase expressed on the surface of the cells produced by the method of claim 13.
- 25 18. A method for improving a target protein, the method comprising the steps of:
- (a) constructing a mutant library of a gene encoding a target protein;
 - (b) constructing a gene recombinant which contains the gene mutant library and a *fadL* gene to express the mutant of the target protein in a form fused with a FadL protein;
- 30

- (c) transforming a host cell with either the gene recombinant or a vector containing the gene recombinant, the host cell being selected from the group consisting of gram negative bacteria, gram positive bacteria, actinomyces, yeasts and molds;
 - 5 (d) culturing the transformed host cells to express the gene mutant library on the cell surface; and
 - (e) screening the cells on which a target protein with improved characteristics have been expressed.
- 10 19. The method for improving a target protein according to claim 18, wherein the screening step is performed by using any one selected from the group consisting of an activity of the target protein, a protein recognizing a substance labeled to the target protein, a labeled ligand binding to the target protein, and an antibody binding specifically to the target protein.

1/2

FIG. 1



2/2

FIG. 2

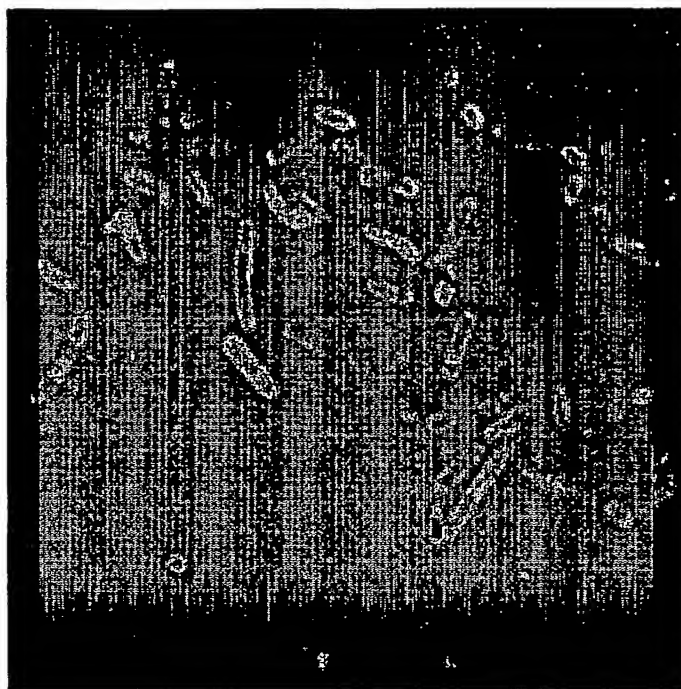
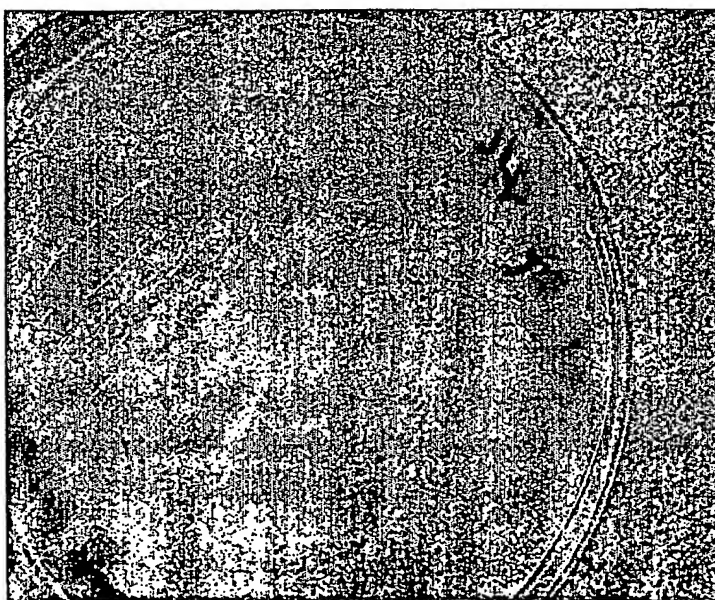


FIG. 3



Sequence Listing

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<151> 2004-06-17

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<223> PCR primer

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VIII-5-1	Declaration: Non-prejudicial disclosure or exceptions to lack of novelty Declaration as to non-prejudicial disclosures or exceptions to lack of novelty (Rules 4.17(v) and 51bis.1(a)(v)): Name (LAST, First)	in relation to this international application KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY declares that the subject matter claimed in this international application was disclosed as follows:
VIII-5-1(I))	Kind of disclosure:	publication
VIII-5-1(I) I)	Date of disclosure:	16 April 2004 (16.04.2004)
VIII-5-1(I) II)	Title of disclosure:	Cell surface display of lipase using Escherichia coli FadL as an anchoring motif
VIII-5-1(I v)	Place of disclosure:	
VIII-5-1(I v)	This declaration is made for the purposes of:	all designations

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2004/002181

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/70, C12N 15/31, C12N 15/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKIPASS, WPI, USPTO, PAJ, PubMed "fadL, Escherichia coli, outer membrane protein, expression vector, display, lipase, etc."

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Biol. Chem., Vol.268, No.21, 25 Jul. 1993, pp.15469-15476, Kumar, GB, Black, PN: "Bacterial long-chain fatty acid transport. Identification of amino acid residues within the outer membrane protein FadL required for activity"	1 - 19
Y	Arch. Biochem. Biophys., Vol.377, No.2, 2000, pp.324-333, Cristalli, G. et al.: "The amino-terminal region of the long-chain fatty acid transport protein FadL contains an externally exposed domain required for bacteriophage T2 binding"	1 - 19
Y	KR 2003-81548 A (Korea Advanced Institute of Science and Technology) 22 Oct. 2003 see the whole document	1 - 19
Y	J. Biotechnol., Vol.96, No.2, 2002, pp.129-154, Samuelson, P. et al.: "Display of proteins on bacteria"	1 - 19
Y	KR 2000-56440 A (Korea Advanced Institute of Science and Technology) 15 Sep. 2001 see the whole document	1 - 19
Y	KR 2003-14959 A (Korea Advanced Institute of Science and Technology) 20 Feb. 2003 see the whole document	1 - 19

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

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Date of the actual completion of the international search

22 MARCH 2005 (22.03.2005)

Date of mailing of the international search report

22 MARCH 2005 (22.03.2005)

Name and mailing address of the ISA/KR



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Authorized officer

SHIN, Kyeong A

Telephone No. 82-42-481-5589



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2004/002181

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



in written format



in computer readable form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in computer readable form



furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2004/002181

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
KR 2000-56440 A	15 Sep. 2000	US 6274345 B1	14 Aug. 2001
KR 2003-14959 A	20 Feb. 2003	WO 03/16538 A1	27 Feb. 2003
		US 2005-19857 A1	27 Jan. 2005
		CN 1466628 A	07 Jan. 2004

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